First trimester embryo-fetoscopic and ultrasound-guided fetal blood sampling for \textit{ex vivo} viral transduction of cultured human fetal mesenchymal stem cells

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BACKGROUND: Intrauterine stem cell transplantation is a promising approach for early onset genetic diseases. However, its utility is limited by the development of the fetal immune system after 14 weeks gestation. An \textit{ex vivo} gene therapy approach targeting autologous first trimester stem cells to replace the missing or defective gene product should overcome this barrier. We investigated the feasibility of harvesting circulating first trimester human fetal mesenchymal stem cells (hfMSCs) for \textit{ex vivo} gene therapy. METHODS: Thin-gauge embryo-fetoscopic-directed or ultrasound-guided blood sampling (FBS) was performed in 18 pre-termination fetuses at a mean of 10.10 (range 7.12 to 13.14) weeks gestation through extra-fetal vessels. Harvested blood was plated for isolation of hfMSC and transduced by lentiviruses. RESULTS: FBS was successful in 12/18 procedures (67%). Success rates were comparable in fetoscopic (4/6) and ultrasound-guided (8/12) procedures, but procedural time was shorter in the ultrasound-guided arm ($P < 0.01$). Fetal bradycardia occurred post-FBS in 33% and 25% of fetoscopic and ultrasound cases, respectively, 5 min post-procedure. hfMSCs were isolated in two-thirds of cases, with high efficiency lentiviral transduction achieved without affecting short-term cell renewal. CONCLUSIONS: This phase-one study demonstrates the feasibility of the \textit{ex vivo} fetal gene therapy approach, in which harvested hfMSCs are genetically manipulated prior to infusion back into the fetus where they should engraft and home to injured tissues. The fetal \textit{ex vivo} gene therapy paradigm is also of relevance to haemopoietic stem cells to treat inherited haematological diseases. Optimization of stem cell harvest and longer-term safety is required before translation into clinical trials in ongoing pregnancies.

\textit{Keywords}: fetoscopy; percutaneous umbilical blood sampling; fetal blood sampling; mesenchymal stem cells; gene therapy

Introduction

Genetic diseases with intrauterine onset can lead to permanent neural or musculoskeletal damage in early life. Examples include the skeletal dysplasias, inborn errors of metabolism and some muscular dystrophies. To prevent this, intrauterine stem cell transplantation has been proposed. This has several advantages over a post-natal approach, including the immunological naïveté of the early gestation fetus, avoidance of ablative preconditioning therapies required for post-natal bone marrow transplantation and intervention before irreversible end-organ damage occurs (Flake and Zanjani, 1999; Flake, 2004).

Clinical success with transplanted allogeneic cells, however, has been largely confined to immune deficiency syndromes (Muench, 2005), which can instead be treated post-natally. Failure in other conditions has been attributed to immune rejection of transplanted allogeneic stem cells after 14 weeks gestation (Renda \textit{et al}., 2000a,b). An alternative approach to intrauterine therapy involves gene therapy with integrating vectors, which has been successful in proof-of-principle studies in animal models of Crigler–Najjar disease (Seppen \textit{et al}., 2003), haemophilia B (Waddington \textit{et al}., 2004) and Leber’s congenital amaurosis (Dejneka \textit{et al}., 2004). However, the risk of unintentional transduction of the germ line and maternal tissue through trans-placental passage of vectors remains a barrier to clinical application. The use of autologous cells for \textit{ex vivo} gene therapy to deliver the missing or defective
protein before 14 weeks gestation should largely overcome this hurdle. Post-natal clinical trials (Aiuti et al., 2002; Hacein-Bey-Ahina et al., 2002; Gaspar et al., 2004; Ott et al., 2006) have validated this approach in children, but it has yet to be applied to the fetus, either clinically or in model systems.

In humans, primitive fetal stem cells (hfMSCs) circulate in high frequencies during the first trimester (<14 weeks), when they can be readily isolated and expanded. Under permissive conditions, they differentiate into fat, bone, cartilage and skeletal muscle (Campagnoli et al., 2001; Chan et al., 2006) and can be efficiently transduced by standard onco-retroviral and lentiviral vectors (Chan et al., 2005). When transplanted into fetal murine models of muscle, renal and skeletal disease, hfMSC demonstrated wide-spread engraftment, site specific differentiation and preferential homing into areas of tissue injury, with improvement in the resulting phenotype (Chan et al., 2007; Guillot et al., 2008a,b).

The development of ex vivo gene therapy is dependent on the ability to acquire the target cells, in this case hfMSC, reliably and safely. Fetal blood sampling (FBS) needs to be performed before 14 weeks of gestation, as hfMSC become infrequent and difficult to isolate thereafter. There also needs to be sufficient time to expand, effect gene transfer of the missing or defective gene product and subsequently re-infuse the cells before the onset of irreversible tissue damage. Although techniques such as ultrasound-guided cannulation of umbilical or fetal intrahepatic vessels from 18 weeks gestation are established obstetric procedures (Nicolini et al., 1988; Daffos, 1989), there have been only a few limited reports of FBS at earlier gestations, and the isolation of stem cells through extra-fetal vessels in the first trimester has not been reported.

To date, isolation of circulating hfMSC in the first trimester fetus has been performed by cardiocentesis, which precludes its use in on-going pregnancies. The development of hfMSC for autologous use requires the harvest of a tiny volume of fetal blood by less invasive methods. Here, we investigated the utility of thin-gauge fetoscopic and ultrasound-guided FBS during the first trimester of pregnancy for the isolation and viral transduction of hfMSC for intrauterine ex vivo gene therapy applications.

Materials and Methods

Ethics and patient recruitment
A prospective study of first trimester thin-gauge fetoscopic or ultrasound-guided FBS was conducted in women undergoing clinically indicated termination of pregnancy. FBS procedures and isolation of fetal stem cells for viral transduction were approved by the Research Ethics Committee (Hammersmith and Queen Charlotte’s Hospitals) in compliance with national guidelines regarding the separation of clinical and research consent for collection of fetal tissue for research purposes (Polkinghorne, 1989).

Women between the ages of 18 and 40 years admitted for clinically indicated termination of pregnancy were approached for participation in this study. Exclusions included medical co-morbidities such as bleeding diathesis or cardio-pulmonary disease. Patients were approached about both procedures without randomization, with those reluctant to undergo the wider bore fetoscopic procedure having the option of consenting to the ultrasound-guided needleling procedure only. Written informed consent was obtained in all cases.

Procedures
An Acuson Sequoia 512™ ultrasound machine using a 4.5 MHz curvilinear transducer was used to guide both invasive procedures and monitor the fetal heart rate and any post-procedural bleeding, which was visualized as streaming from the vessels. After induction of general anaesthesia, the crown rump length and fetoplacental anatomical relationships were determined as detailed above. The location of the target vessels was next identified to guide the entry point of the sampling device. Upon cannulation of a cord vessel, a small volume of fetal blood was aspirated into a 1 ml graduated syringe. Aspiration of a minimal volume of 50 µl was attempted, with larger volumes withdrawn in some of the later gestational age cases to test for any effects on short-term fetal outcome. Fetal heart rate was determined ultrasonically over 1 min before and 5 min after the procedure by direct visualization of the fetal heart with the use of a digital stopwatch by an assistant. Digital ultrasound clips were captured and stored as compressed video in MPEG-1 (Motion Picture Expert Group).

For embryo-fetoscopy, a 1 mm diameter fibre-optic fetoscope (11 510, Karl Storz) comprising 10 000 optical fibres with a 70° viewing angle was inserted under ultrasound guidance, together with a 21G operator sheath (11 510KD, Karl Storz) with a side port to introduce a 26G hypodermic puncture needle (11 510KC, Karl Storz) was used. A 300 kW Xenon light source was used for endoscopic visualization with images recorded on an Aida digital video capture system (AIDA, Karl Storz). The umbilical vessels were targeted in a free loop of cord or more usually at the fixed end of the placental cord insertion site, aiming to cannulate the umbilical vein rather than an artery. On withdrawing the needle, the entry site was directly observed for the cessation of fetal bleeding for up to a minute before withdrawing the scope.

For ultrasound-guided FBS, three different approaches were used as appropriate: aplatental, transamniotic and transplacental. A siliconized 20G sampling needle was used (Cook Ltd, UK) as for ultrasound-guided sampling in later gestation, and as used for first trimester cardiocenteses (Shannon et al., 1998; Campagnoli et al., 2001). The vessels targeted included the chorionic plate vein, or umbilical vein, either in a free loop of umbilical cord or at the placental cord insertion.

All procedures were performed by one of the two maternal fetal medicine specialists (NMF, SK).

Isolation of hfMSC
Aspirated fetal blood was plated in 100 mm dishes at 10⁶ nucleated cells per ml to isolate and expand hfMSC as previously reported (Campagnoli et al., 2001). The identity of putative hfMSC was confirmed through colony forming unit (CFU) assays, immunophenotype and multi-lineage differentiation as detailed elsewhere (Chan et al., 2005, 2006). hfMSC at passages 3–7 were used for viral transduction experiments.
**Viral transduction of stem cells**

Two different lentiviral constructs were used to transduce hMSC; either (i) a bi-directional lentivirus pRRL.SIN18.cPPT.fLuc.mCMV.hPGK.eGFP.WPRE encoding the firefly luciferase (fLuc) and enhanced green fluorescence protein (eGFP) transgenes driven by ubiquitous promoters minimal cytomegalovirus (mCMV) and human Phosphogluco-kinase (hPGK) (Amendola et al., 2005), or; (ii) the lentiviral vector pWPTS.nlsLucZ (http://tronolah.epfl.ch/) driving the expression of nuclear localizing LacZ (nlsZ) through an ubiquitous promoter EF1α (elongation factor 1α) (see Figures). Generation of lentiviruses through triple transfection of 293T human embryonic kidney cell lines, and transduction of hMSC was done as previously described (Chan et al., 2005; Kurata et al., 2007).

Analysis of LacZ expression was done by X-Gal staining (Sigma, UK), counting six low powered fields (X4) on light microscopy 5 days post-transduction (a range of 126–169 cells were enumerated, totalling 847 cells). eGFP expression was assessed by flow cytometry (FACS Calibur, Becton-Dickinson, UK) by excitation at 488 nm and detection through the FL1 channel at 530 nm. Luciferase activity was assessed by immunostaining for fLuc (Abcam, UK) after fixing the cells in 4% paraformaldehyde at room temperature for 1 h and blocking the cells with non-serum protein block (DAKO X0909, UK). After permeabilization with 0.2% Triton X, incubation with mouse anti-fLuc (1:100) at room temperature for 1 h, and subsequent incubation with anti-mouse immunoglobulin G conjugated with alexaflour594 (1:100, Invitrogen, UK), slides were treated with 4′,6-diamidino-2-phenylindole (DAPI, H-1200, Vector Laboratories, UK). Thereafter, the slides were analysed by epifluorescence microscopy (Zeiss Axioscope I), where visualization of eGFP was done concurrently, and images captured by a cooled charge-coupled device camera and reviewed in Quips m-FISH software (Vysis, UK). The functional activity of luciferase was assayed after adding the substrate luciferin to the transduced cells, and imaging with Xenogen livingimage v2.50 (Xenogen).

**Statistics**

Parametric variables were expressed as mean and SD and compared by Student’s *t*-test. A *P*-value of < 0.05 was considered significant.

**Results**

A total of 18 patients were studied at a median gestation of 10⁴⁺⁰ (range 7⁻² to 13⁻⁴) weeks, with 6 in the fetoscopic arm and 12 in the ultrasound-guided arm.

**Fetoscopic-directed FBS**

Six fetoscopies were performed in pregnancies between 9⁻⁴ and 13⁻⁴ weeks gestation (Table I). Embryo-fetoscopy allowed visualization of various parts of the fetal anatomy, with good views of both upper and lower limbs, external genitalia, abdomen and head (Fig. 1). Funipuncture and aspiration of fetal blood was successfully performed in four of six cases, with both failures occurring at the first two fetoscopic FBS attempts with procedure times of more than 10 min (15 and 12 min). In these two cases, the amnion collapsed within the amniotic cavity due to amniotic fluid draining out of the amniotic cavity into the extra-amniotic space, obscuring the view of the umbilical vessels and hence precluding funipuncture itself. In the latter four cases, operating time was shorter (5–10 min) and FBS was attempted immediately after identification of the target vessel, with the fetal circulation successfully accessed at the placental cord insertion (two out of four cases, Fig. 2A) or a free loop of umbilical cord (two out of four cases, Fig. 2B), aspirating between 50 and 300 μl of fetal blood (Table I).

In all cases, only one uterine entry was required, whereas the number of attempts at cannulating the target vessel varied between 1 and 5. There were two cases of fetal bradycardia 5 min after the procedure (33%) (Table I). All fetoscope insertions were performed aplacently except the last case, where the placenta was sited anteriorly and the presence of overlying bowel did not allow for a lateral entry approach to avoid the placenta. The resultant placental bleeding obscured the view somewhat, although FBS was successfully completed. In all cases, where FBS was successful, bleeding from the cord was observed upon withdrawal of the sampling needle, which resolved spontaneously within 1 min (Fig. 2C–F).

**Ultrasound-guided FBS**

Ultrasound-guided FBS was successful in 8 of 12 cases (median 10⁺³ weeks, range 7⁻² – 13⁻²), including collections from both chorionic plate vessels and the umbilical cord at either its insertion or a free loop of cord (Fig. 3). Of the four cases where FBS failed, there was a ‘dry tap’ where the needle tip looked to be within the umbilical vessel, yet no fetal blood could be withdrawn into the syringe. The volumes aspirated in successful cases ranged between 50 and 300 μl from fetuses between 7⁻² and 13⁻² weeks (Table II). The mean time taken for the procedure was less than that in the fetoscopic group at 5.9 ± 1.8 min versus 9.3 ± 3.5 min (*P = 0.01)*.

Only one uterine entry of the sampling needle was required, but the median number of attempted vessel cannulations was 2 (range 1–7). There was ultrasonic evidence of bleeding from

**Table I. Fetoscopic FBS.**

<table>
<thead>
<tr>
<th>Gest</th>
<th>Site</th>
<th>Placenta</th>
<th>FBS (μl)</th>
<th>Atmp</th>
<th>FHR pre</th>
<th>FHR post</th>
<th>MSC culture</th>
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<tr>
<td>1</td>
<td>13⁻⁰⁰</td>
<td>UC</td>
<td>Post</td>
<td>nil</td>
<td>4</td>
<td>135</td>
<td>145</td>
</tr>
<tr>
<td>2</td>
<td>9⁻⁰⁰</td>
<td>UC</td>
<td>Post</td>
<td>nil</td>
<td>5</td>
<td>140</td>
<td>140</td>
</tr>
<tr>
<td>3</td>
<td>12⁻⁰⁰</td>
<td>PCI</td>
<td>Ant</td>
<td>300</td>
<td>3</td>
<td>130</td>
<td>80</td>
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<tr>
<td>4</td>
<td>9⁻⁶</td>
<td>PCI</td>
<td>Post</td>
<td>100</td>
<td>2</td>
<td>140</td>
<td>100</td>
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<tr>
<td>5</td>
<td>13⁻⁴⁴</td>
<td>UC</td>
<td>Post</td>
<td>200</td>
<td>1</td>
<td>140</td>
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<tr>
<td>6</td>
<td>10⁻⁴⁴</td>
<td>UC</td>
<td>Ant</td>
<td>50</td>
<td>2</td>
<td>140</td>
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<td>50–300</td>
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</tbody>
</table>

Six cases were attempted between 9⁻⁴ and 13⁻⁴ weeks of gestation (Gest). UC, umbilical cord; PCI, placental cord insertion; Ant, anterior; Post, posterior; Atmp, attempts; ND, not done; FHR, fetal heart rate; MSC, mesenchymal stem cells.
the puncture site of the umbilical cord (streaming from the vessel under ultrasound imaging) in four of eight successful cases (50%), all of which involved transamniotic approaches. As with the fetoscopic approach, bleeding resolved spontaneously within a minute. Fetal bradycardia 5 min after FBS occurred in 3 of 12 cases (25%) (Table II).

**Isolation of hfMSC from first trimester fetal blood**

When fetoscopically directed \((n = 3)\) and ultrasound-guided \((n = 6)\) fetal blood samples were plated in hfMSC growth media, adherent spindle-shaped cells resembling hfMSC appeared in two of three (67%) fetoscopic FBS and four out of six (67%) ultrasound-guided FBS samples after 72 h (Table I and II). These cultures reached subconfluence within 7–10 days of plating. The doubling time of hfMSC cultures was \(30.4 \pm 2.1\) h \((n = 3)\), with each passage resulting in 3–4 population doublings. Immunophenotyping confirmed their non-haemopoietic non-endothelial phenotype, being negative for CD34, CD45, CD14, CD31 and von Willebrand factor, while expressing mesenchymal type markers CD105, SH3,

Figure 1: Fetoscopy allowed visualization of fetal anatomy such as the head, upper limbs (A and B) and lower limbs (C) at 9 weeks gestation. The fingers (D) external genitalia (E, male) and physiological bowel herniation (F) were clearly seen.
SH4 and vimentin and cell adhesion molecules CD29, α2, α4 and α5 integrin (CD49b, CD49d and CD49e), CD44, CD106 (VCAM-1), laminin and fibronectin. hfMSC did not express HLA-II and had low levels of HLA-I. Under permissive media, they differentiated readily into osteoblasts and adipocytes as previously reported (Campagnoli et al., 2001; Chan et al., 2005), confirming their identity as bona fide hfMSC (data not shown). Successful hfMSC cultures were achieved from sample volumes as low as 50 μl fetal blood (Table II).

**Lentiviral transduction of hfMSC**

Isolated hfMSCs were transduced with either the nlsZ or the bi-directional luciferase and eGFP lentiviral constructs with efficiencies of >90% for both vectors (Fig. 4), with evidence of transgene expression after X-Gal Staining (Fig. 4C) or

**Figure 2:** Fetoscopic access to the fetal circulation was achieved through needling the umbilical vein where the cord inserts into the placenta (red arrow) (A) or at a free loop of umbilical cord where the placental cord insertion was not accessible due to fetal lie or an anterior placenta (B). Bleeding from the cannulated vessels occurred in all the cases after withdrawal of the sampling needle (it can be still observed within the field of view) as seen here in sequence (C–F), which lasted for under 1 min in all cases.

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immunostaining for luciferase and GFP expression under epi-fluorescence microscopy (Fig. 4D–G). Functional activity of luciferase was confirmed by the detection of bioluminescence after addition of the luciferin substrate into the cell culture with a Xenogen bioluminescence imager (Fig. 4I). Transduction of hfMSC did not affect their capacity to expand in culture.

Figure 3: (A) Ultrasound-guided FBS of chorionic plate vessels close to the umbilical cord insertion in a fetus at 13+2 weeks gestation, with target vessels shown on colour flow Doppler. (B and C) A transamniotic approach with cannulation of a free loop of umbilical cord at 11+0 weeks gestation is shown here with colour flow Doppler (B) and with the sampling needle within the cord (C). A transplacental approach was also feasible, with FBS performed at the placental cord insertion after transgressing the placenta in a fetus at 10+3 gestation (D and E).
Discussion

Although intrauterine cell therapy holds tremendous potential in the treatment of various genetic diseases, its effectiveness has been limited by the development of the fetal immune system which seems capable of rejecting allogeneic cells/foreign transgenes after 14 weeks of gestation (Haynes, 1984; Sprent, 1995; Goodnow, 1996). This suggests a critical window in which either stem cell transplantation or ex vivo gene therapy can be applied, where the delivered transgene/antigen can be presented for central thymic processing, and thus the induction of tolerance. In this study, we demonstrated the feasibility of harvesting first trimester fetal blood-derived MSC through both thin-gauge embryo-fetoscopy and ultrasound-guided FBS with comparable success. This was possible as early as 7–12 weeks of gestation, with a two-thirds success rate for both fetoscopic and ultrasound-guided approaches. Overall harvest of hfMSC was successful in 67% of cases attempted (n = 9). In addition, harvested hfMSCs were efficiently transduced with standard lentiviruses without affecting their expansion capacity. This study validated the ex vivo gene therapy approach, by obtaining useful quantities of first trimester fetal blood for the ex vivo genetic manipulation of isolated hfMSC. Downstream these can then be infused back into the fetus where they are expected to engraft widely and home to injured tissues, as previously reported in murine models (Chan et al., 2007; Guillot et al., 2008a,b).

Fetoscopic-directed FBS

Following the development of thin-gauge endoscopes, several groups have reported its utility for prenatal diagnostic purposes, including a small number of attempts to access the fetal circulation. Surbek et al. reported successful aspiration of 100 μl of fetal blood from two out of three cases (67%) at around 10 weeks gestation, but did not indicate which umbilical vessel, or the part of the umbilical cord, was targeted (Surbek et al., 2000). Miliou-Pauleskou et al. reported on aspiration of fetal blood from either the chorionic plate vessels or umbilical cord in 10 out of 14 cases (71%) using similar equipment at 9–12 weeks gestation, but did not report the volume of fetal blood obtained (Miliou-Pauleskou et al., 2001). Neither group attempted to isolate stem cells from these samples. Their results however, were similar to our success rates with obtaining fetal blood. In performing the first two cases, we attempted funipuncture after a prolonged period surveying fetal anatomy, leading to the collapse of the amnion which greatly reduced visualisation of the umbilical cord. In the subsequent four cases, we performed funipuncture immediately after four visualising the target site, with a resultant shorter procedural time.

Ultrasound-guided FBS

The development of percutaneous ultrasound-guided FBS techniques in the early 1980s offered a simpler alternative to the fetoscopic approach and hence became the standard method in most maternal–fetal medicine units for accessing the fetal circulation (Hobbins et al., 1985; Daffos, 1989). While most of the literature on success and fetal loss rates from FBS are for procedures performed at or after 18 weeks gestation, experience at lower gestations remains limited.

Orlandi et al. reported a series of 500 ultrasound-guided FBS for prenatal diagnosis between 12 and 19 weeks of gestation, using a 25G sampling needle. Forty-two were performed between 12 and 14 weeks with a procedure-related fetal loss rate of 4.8% (Orlandi et al., 1990). More recently, Lam et al. performed ultrasound-guided FBS with a 26G sampling needle at 12–14 weeks gestation in fetuses with suspected α-thalassaemia. FBS was successfully achieved in 97% (57 of 59) at the placental cord insertion, albeit with a procedure-related loss rate of 8% (5/59) (Lam and Tang, 2000). In that series, all sampled fetuses were anaemic with haemoglobin Bart’s disease which may account for the higher loss rate compared with Orlandi et al. (1990).

The above results, however, cannot be reliably extrapolated to earlier gestations (<12 weeks), when the size of the fetal vessels is extremely small. For example, the umbilical vein diameter increases from 2.0 to 4.1 mm between 12 and 18 weeks gestation, as measured by ultrasonography (Weissman et al., 1994). Despite this, we managed to achieve successful FBS with a 20G needle, as early as 7–12 weeks gestation, with three quarters of cases performed before 12 weeks, with fetal blood successfully aspirated in six of these nine (67%). We observed comparable bradycardia rates of 25% compared with 20% reported by Lam et al.’s group (Lam and Tang,
This was considerably higher than that reported by Orlandi et al. (1990) of 2.5% for cases between 12 and 19 weeks gestation, which might be due to our lower gestations (median of 10\(\pm\)3 weeks of gestation), or our use of a larger sampling needle itself. The lower incidence of cord bleeding observed with an ultrasound-guided versus a fetoscopic approach (50% versus 100%) might be due to the failure of the ultrasound machine to detect minor occurrences of low-flow bleeding post-FBS, which are likely to be more directly visualized at fetoscopy. However, all bleeding after the ultrasound-guided samplings followed transamniotic approaches, so a more likely explanation is that bleeding complicates all procedures, but these are masked by intraplacental bleeding or tamponading with transplacental approaches, as happens with clinically indicated procedures at later gestations.

Comparisons between fetoscopic and ultrasound-guided approaches
Compared with the fetoscopic approach, ultrasound-guided FBS was simpler, significantly faster and less equipment-intensive. In addition, experience in this and related ultrasound-guided needling techniques at later gestational ages already exists in most specialist feto-maternal centres. On the other hand, it was more difficult to target accurately under ultrasound guidance the relatively small umbilical vein at this gestation, as can be done under direct fetoscopic visualization. An analysis of blood gases from the sample would have given some confirmation as to the vessel sampled; however, it was not performed due to the small volumes of fetal blood aspirated. The vein was the target vessel in all cases and, although we cannot be sure, it is the more likely as the larger of the three cord vessels.
Despite the use of different needle sizes in the two groups, with a smaller 26G needle in the fetoscopic group and a larger 20G needle for the ultrasound-guided group, we observed similar success rates of FBS as well as rates of bradycardia.

**Fetal-placental blood volume and frequency of hfMSC**

In order to utilize hfMSC as a target cell type for ex vivo gene therapy, a sufficient volume of fetal blood is required for successful isolation and expansion of hfMSC in vivo. This on the other hand has to be balanced with the risk of inducing hypovolaemic circulatory embarrassment in the fetus. Although the volume of circulating blood in the early gestation fetus is not known, an assumption can be made based on previous data on human feto-placental blood volume between 18 and 31 weeks gestation, which showed a gradual drop in circulating blood volume per unit of fetal weight from 117 ml/kg at 18 weeks to 93 ml/kg at 31 weeks, with a mean of 101 ml/kg (Nicolaides et al., 1987), in keeping with data from sheep studies (Brace, 1983). Furthermore, data gathered from chronically cannulated fetal-sheep models suggest a loss of 15% of circulatory blood volume is tolerated reasonably well (Macdonald et al., 1980). We therefore assumed that it would be possible to aspirate up to 60 μl of blood at 8 weeks of gestation, accounting for 10% of fetal weight (Brenner et al., 1976), without causing circulatory embarrassment. This small volume of blood is adequate for isolation of hfMSC, which circulate at a frequency of 6.9 ± 1.0 CFU per 10 μl of fetal blood (Campagnoli et al., 2001), as demonstrated in one case where hfMSCs were isolated from 50 μl fetal blood plated (Table II). Fifty microlitres of fetal blood should generate 35 hfMSC colonies. Given that hfMSC cultures double every 30 h, this should result in the generation of 10^6 cells in 19 days, and 10^9 cells by 31 days, which supports their use as a target for gene transfer.

In this study, we attempted to aspirate at least 50 μl of fetal blood for the purpose of hfMSC isolation, whereas in some cases, we attempted a larger volume to test if there was a relationship between the volume aspirated and the occurrence of fetal bradycardia. Although a clear correlation was not established, all the cases of bradycardia occurred where >50 μl of fetal blood was aspirated.

**Isolation of hfMSC**

hfMSCs were successfully cultured in only two-thirds of plated samples, which was comparable with that achieved from cardiocentesis samples (data not shown). Although the small volume of blood aspirated here may result in some technical difficulties with clotting of the sample, the same plating density was used throughout. Aside from fetal blood hfMSC, which only circulate in high frequencies between 8 and 14 weeks gestation (Campagnoli et al., 2001), other sources of autologous hfMSC may have a role to play in ex vivo gene therapy. Fetal liver, which is a rich source of hfMSC during the first and second trimester, has been successfully isolated by several groups (Campagnoli et al., 2001; Gotherstrom et al., 2003; In’t Anker et al., 2003a,b). Sampling the fetal liver, however, is more invasive, leading to a fetal loss rate of 33% when attempted in a late first trimester fetal lamb paradigm (Surbek et al., 2002). The use of hfMSC derived from amniotic fluid (In’t Anker et al., 2003a,b; Tsai et al., 2004; De Coppi et al., 2007) would be less invasive. However, its isolation has only been described in second-trimester samples thus far, which is too late for central thymic antigen presentation, and hence this approach will still be subjected to immune rejection of the delivered transgene by the immune-competent fetus. The isolation of first trimester chorion villi or amniotic fluid derived MSC in future may overcome this gestation-specific hurdle.

**Safety of first trimester FBS**

In this study, it was not possible to gather long-term outcome data, due to the nature of this study performed necessarily in patients undergoing termination of pregnancy. Although Orlandi et al. reported a loss rate of 4.8% and Lau et al. 8.0% (all fetuses had hydrops) between 12 and 14 weeks of gestation in ongoing pregnancies (Orlandi et al., 1990; Lam and Tang, 2000), it remains to be determined what the complication rates will be before 12 weeks gestation. This rate is likely to be higher at earlier gestations, though it may still be acceptable in situations where the alternative is termination of an affected pregnancy.

**Conclusion**

Ex vivo intrauterine gene therapy has considerable potential against various inherited genetic disorders, in particular when performed during a critical window (<14 weeks gestation) which allows central tolerance to be developed towards the absent protein/gene product, and prevent rejection, which unto now has been the main barrier to clinical translation (Shields et al., 2002; Muench, 2005). We have shown here that fetoscopic-directed or ultrasound-guided FBS through extra-fetal vessels in the first trimester allows the harvest of hfMSC and their eventual genetic manipulation. This approach allows targeted delivery of transgenes, which could be applied not only for mesenchymal deficiency disorders, such as skeletal dysplasias and muscular dystrophies, but also for enzyme deficiency disorders, such as the mucopolysaccharidoses, or as a cell factory for delivering clotting factors (Bigger et al., 2006). The techniques mentioned here will also enable the harvest of primitive fetal haemopoietic stem cells, which are present in high frequencies (5% versus 0.4% at term; Campagnoli et al., 2000), for ex vivo gene therapy relevant to a range of inherited haemopoietic disorders (Muench, 2005). Further optimization of the harvest of stem cells and investigations into the longer-term safety of this procedure will push this field into the clinical realm.

**Supplementary Data**

Supplementary data are available at http://humrep.oxfordjournals.org.

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Sampling and viral transduction of fetal stem cells